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Full Length Research Paper

Modification of membrane lipid peroxidation and antioxidant enzymes activation in transgenic rice resistant to *Rhizoctonia solani*

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Activation of membrane lipid peroxidation and antioxidant enzymes were investigated in transgenic rice (*Oryza sativa* L.) lines J6 and J61 expressing the rice chitinase gene (*RCH10*) and the alfalfa β-1,3-glucanase gene (*AGLU1*). Our results show enhanced resistance of both lines to *Rhizoctonia solani*, the causal agent of rice sheath blight disease. While malondialdehyde (MDA) was significantly generated with increasing infection time, maximum production in wild-type (WT) transgenic lines J6 and J61 (44.35, 35.00 and 38.65 µmol/g FW, respectively) occurred after 4 days. Analysis of superoxide dismutase (SOD) activity after 2 days inoculation also revealed distinct differences between WT (34.45 U/mg protein) and the 2 transgenic lines (J6; 45.86 U/mg protein; J61, 71.90 U/mg protein). On the other hand, the activities of catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (GPX), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) were found to be quite similar in all 3 lines. Taken together, these findings suggest that MDA content and SOD activity might play important roles in enhancing pathogen resistance in the transgenic plants. Our study provides a valuable route to analyze the mechanism of transgene-mediated defense in transgenic rice.

Key words: Transgenic rice, resistance, sheath blight, *Rhizoctonia solani*, membrane lipid peroxidation, antioxidant enzymes.

INTRODUCTION

As one of the most important food crops, rice (*Oryza sativa* L.) provides staple diet for more than half of the world population, especially in developing countries (Venu et al., 2007). The crop is constantly confronted with a wide variety of potential pathogens throughout its lifetime, resulting in reduced production. Among them, sheath blight caused by *Rhizoctonia solani* Kühn, a soil-borne fungal pathogen with a broad host range, whose genetic mechanisms of effective plant resistance is yet to be discovered, causes severe damage to the annual rice yield (Pinson et al., 2005). For years, fungicides are routinely used into controlling these fungal diseases, raising considerable concerns on human health and environment. As a result, attentions have been turned to alternative strategies to enhance disease resistance of crops against invading pathogens, via understanding the genetic mechanisms responsible for effective defense response.

In this instant, with the rapid development of modern biotechnology, many pathogen resistant (PR) and PR-like proteins have been identified and introduced successfully into elite varieties (Campbell et al., 2002; Franco, 2007), many of these PR proteins have been found to be chitinases and glucanases, the hydrolytic enzymes that can directly kill fungal pathogens (Shah and Klessig, 1996; Gijzen et al., 2001; Thimmapuram et al., 2001; Park et al., 2004; Hoster et al., 2005; Nakazaki et al., 2006). The availability of PR genes and their antifungal functions has led to their deployment for increasing

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Abbreviations: MDA, Malondialdehyde; **WT,** wild-type; **SOD,** superoxide dismutase; **CAT,** catalase; **APX**, ascorbate peroxidase; **DHAR,** dehydroascorbate reductase; **GR,** glutathione reductase; **GPX,** guaiacol peroxidase.

disease resistance in crops. There are also increasing number of reports on the effects of genetically engineered rice on enhanced resistance to mitigate crop loss imposed by pathogens such as *R. solani* in recent years (Lin et al., 1995; Datta et al., 1999, 2001; Coca et al., 2006; Shah et al., 2009).

Upon pathogen invasion, an oxidative burst is one of the most rapid defense reactions elicited in the plant, which in turn leads to the transient production of high levels of reactive oxygen species (ROS) that include superoxide (O^{2-}), hydrogen peroxide (H_2O_2), and the hydroxyl radical (·OH). ROS produced in the oxidative burst has been demonstrated not only to protect against invading pathogens but to function as signaling molecules to activate plant defense responses in many plant-pathogen interactions (Lamb and Dixon, 1997; Dangl and Jones, 2001; Apel and Hirt, 2004; Aguirre et al., 2005). However, in order to avoid plethora ROS, the plant has evolved to efficiently scavenge these damaging effects by triggering. Such a system involves both enzymatic and non-enzymatic antioxidants, where the enzymatic protective mechanism operates by sequential and simultaneously activating a number of elevation/induction antioxidant enzymes (catalase activity, CAT; superoxide dismutase, SOD; ascorbate peroxidase, APX; guaiacol peroxidase, GPX; glutathione reductase, GR) and non-enzymatic antioxidants, like ascorbate (AsA), glutathione (GSH), flavonoids and carotenoids (Panda, 2007). Because of the complexity of these processes, the mechanisms behind sheath blight resistance are still mainly unknown (Pinson et al., 2005).

Our previous work showed that the transgenic rice (cv. Taipei 309) co-transformed with the rice chitinase (*RCH10*) and alfalfa β-1,3-glucanase genes (*AGLU1*) exhibits enhanced resistance to rice sheath blight under greenhouse condition.

In the present study, we compared *R. solani*-induced lipid peroxidation and antioxidant responses of transgenic against wild-type (WT) rice. Malondialdehyde (MDA) generation was induced by pathogen infection and its amount in transgenic plants was significantly lower than WT ones, with or without pathogen inoculation. SOD activity was significantly stimulated by pathogens in transgenic as compared to WT rice. These observations provide new insights into transgene-mediated resistance involved in plant defense responses.

MATERIALS AND METHODS

Plant culture and treatment

Earlier, the rice chitinase gene (*RCH10*) and alfalfa β-1,3-glucanase gene (*AGLU1*) were tandem-inserted into the transformation vector pBI101 under the control of the 35S promoter with its enhancer sequence to generate double-defense gene expression cassette pZ100. The pZ100 cassette was transformed into rice (cv. Taipei 309) by Agrobacterium-mediated transformation. Two stable transgenic lines (J6 and J61) were obtained. The fifth generation (T5) of J6 and J61 were used as the donor plants. After germination,and grown to the maximum tillering stage to be used for inoculation.

All experiments were conducted under greenhouse conditions. The rice sheath blight pathogen, *R. solani*, was maintained on a potato-dextrose agar plate as described by Pan (1999). On the following day, 5-mm agar disk from the periphery of an actively growing colony of *R. solani*, held in place by sterile 1 cm in length were transferred to PDA in a Petri dish. The plates were incubated at 28°C until the toothpicks were colonized by the pathogen.

Plants were inoculated at tillering stage by placing the 1-cm *R. solani* colonized toothpick into the lowest inner sheath of the main tiller. A new non-colonized toothpick was placed on the same location as a control. After inoculation, the control and inoculated plants were immediately transferred to a growth chamber and kept at 27°C with 90% relative humidity. Leaves were harvested at 0, 24, 48 and 96 h after treatment. Samples were immediately immersed in liquid nitrogen and stored at -80°C for subsequent analysis. A total of 5 plants were sampled for each treatment and time interval.

Determination of malondialdehyde content

Lipid peroxidation was estimated as MDA content, determined according to Madhava and Sresty (2000), with the following modifications: 0.3 g of fresh leaves without the main midrib was homogenized in 2 ml of 10% (w/v) trichloroacetic acid (TCA) solution. The homogenate was centrifuged at 10,000g for 20 min, and then 1 ml of the supernatant was added to 1 ml of 20% TCA containing 0.5% thiobarbituric acid (TBA) and 0.01 ml of butylated hydroxytoluene (BHT) (4% solution in ethanol). The mixture was incubated in boiling water for 20 min, and the reaction was stopped in an ice bath. After that the cooled samples were centrifuged at 10,000 g for 10 min, and the absorbance of the supernatant was recorded at 532 nm and the value for non-specific absorption at 600 nm was subtracted. Loss of membrane integrity was measured from the extinction coefficient as 155 $mmol^{-1}$ cm^{-1}. Results are presented in μmol g^{-1} FW. Experiments were performed in 5 replicates.

Assay of antioxidant enzyme activity

Typically, 0.3 g fresh leaves without the main midrib was homogenized with 2% PVPP and 50 mM HEPES extraction buffer (pH 7.8) containing 0.2 mM EDTA and 2 mM ascorbic acid in a pre-chilled motor and pestle. The homogenate was then centrifuged at 12,000g for 20 min at 4°C. The supernatant was used as the crude extract for enzymatic assays.

Activity of superoxide dismutase (SOD, EC 1.15.1.1) was assayed by measuring the inhibition of photochemical reduction of nitro-blue tetrazolium (NBT) (Stewart and Bewley, 1980). A 3 ml reaction mixture contained 50 mM potassium phosphate buffer (pH 7.8), 13 mM methionine, 2 mM riboflavin and 56 mM NBT and 50 μl enzyme extract. The absorbance of solution was measured at 560 nm. One unit of superoxide dismutase activity was defined as the amount of enzyme that caused half-maximal inhibition of the initial rate of the reaction in the absence of enzyme.

Analysis of guaiacol peroxidase (GPX, EC 1.11.1.7) activity was assayed according to Zhou (2007) as follows: the reaction mixture contained 2.5 ml of 50 mM potassium phosphate buffer (pH 6.1), 1 ml of 1% hydrogen peroxide, 1 ml of 1% guaiacol and 10 to 20 μl crude extract. The increase in absorbance at 420 nm was recorded. Activity was calculated using the extinction coefficient (26.6 $mmol^{-1}$ cm^{-1}). One unit of GPX specific activity was defined as the amount to decompose 1 μmol of hydrogen peroxide min^{-1} mg $protein^{-1}$ under the assay conditions.

Ascorbate peroxidase (APX, EC 1.11.1.11) activity was measured

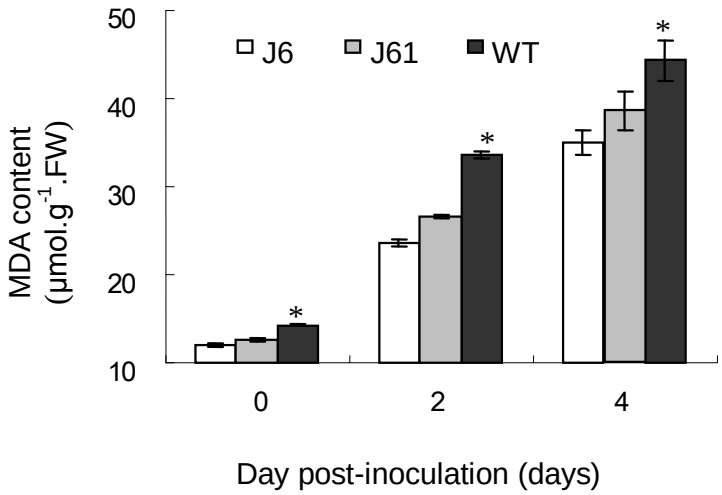

Figure 1. Time-course analysis of MDA in wild-type and transgenic lines. J6: The 6th line of the 5th generation of transformants; J61: 61th line of the 5th generation of transformants; WT: wild-type. Error bars are means ± SD gotten from (n = 5) five biological replicates. An asterisk indicates the significant difference at *P* values <0.05 between the transgenic lines and the control.

by the decrease in absorbance at 290 nm as ascorbate was oxidized (Nakano and Asada, 1981). The assay of dehydroascorbate reductase (DHAR, EC 1.8.5.1) activity was germination, 5 to 6 days old seedlings were transferred to nutrient carried out by measuring the increase in absorbance at 265 nm due to reduced ascorbate formation (Nakano and Asada, 1981).

Glutathione reductase (GR, EC 1.6.4.2) was assayed according to Foyer and Halliwell (1976) by tracing the decrease in absorbance at 340 nm due to NADPH oxidation. Catalase activity (CAT, EC 1.11.1.6) was determined by the method of Cakmak and Marschner (1992).

Protein concentration of the crude extract was determined according to Bradford (1976).

Statistical analysis

All treatments were conducted in a randomized complete block design. For each enzymatic analysis, 5 independent tissue samples were used. Data was expressed as mean ± standard deviation (SD). Duncan's multiple-range test (SSR) was used with the data processing system (DPS) statistical software package (Tang and Feng, 1997).

RESULTS

Effect of transgenes on lipid peroxidation

Lipid peroxidation was examined as a function of oxidative stress, estimated as MDA produced. MDA, a decomposition product of polyunsaturated fatty acid hydroperoxides, is produced upon oxidative damage. As shown in Figure 1, MDA gradually increased after pathogen invasion in all 3 plants, with a significant difference ($P < 0.05$) observed between WT and transgenic plants. Interestingly, a significant difference ($P < 0.05$) was observed between the 2 transgenic lines J6 and J61, at only 2 days after infection. Overall, maximum MDA production was reached in WT (44.35 μmol/g FW), J6 (35.00 μmol/g FW) and J61 (38.65 μmol/g FW) after 4 days post-inoculation.

Effect of transgenes on SOD activity

SOD catalyzes the breakdown of superoxide anions to hydrogen peroxide and dioxygen. It plays a critical role in host preliminary defense against pathogens invasion. Effect of transgenes on SOD activity is shown in Figure 2. At 0 day of infection, only very little increase was observed in transgenic lines to WT. However, a sharp increase, with a significant difference ($P < 0.05$) was detected in J6 and J61 after 2 days inoculation. In contrast, the SOD activity of all declined drastically which was reduced by 28.9, 53.9 and 44.1%, respectively after 4 days inoculation. These results implied that transgenes enhanced resistance to *R. solani* by increasing SOD production.

Effect of transgenes on CAT and GPX activities

CAT and GPX are involved in the detoxification of H_2O_2, thereby preventing the formation of ·OH radicals. As shown in Figures 3 and 4, CAT and GPX activities gradually increased both in WT and transgenic plants after pathogen invasion with no significant difference ($P < 0.05$) observed among transgenic lines J6, J61 and WT. However, after 4 days, transgenic lines responded significantly ($P < 0.05$) to *R. solani* (Figure 4).

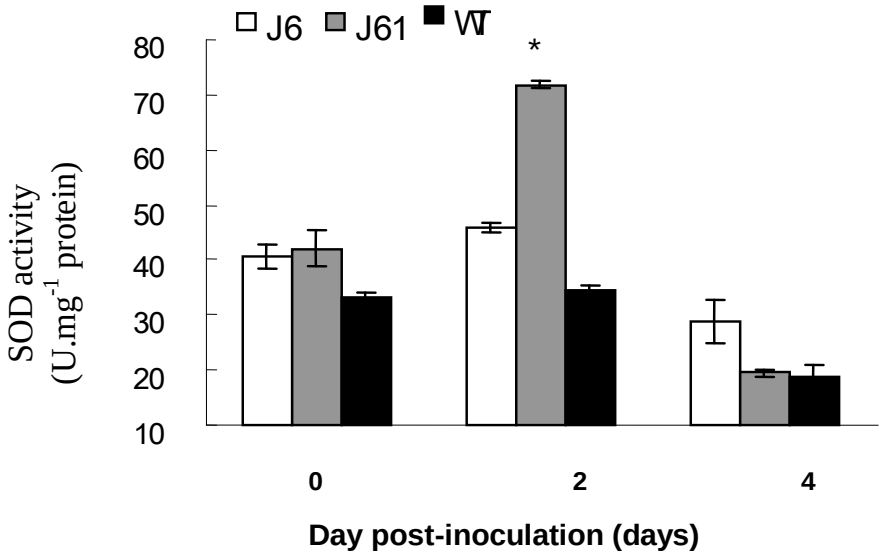

Figure 2. Time-course analysis of SOD activity in wild-type and transgenic lines. J6: The 6th line of the 5th generation of transformants; J61: 61th line of the 5th generation of transformants; WT: wild-type. Error bars are means ± SD gotten from (n = 5) five biological replicates. Asterisk indicates the significant difference at *P* values <0.05 between the transgenic lines and the control.

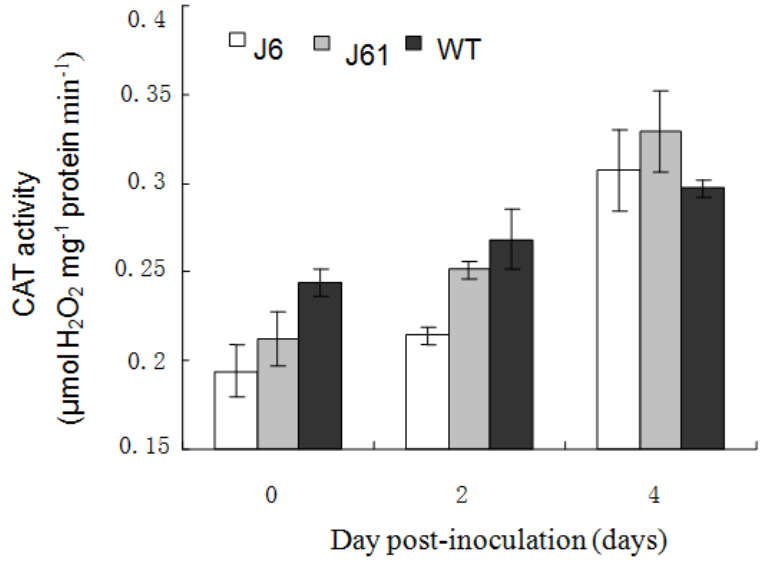

Figure 3. Time-course analysis of CAT activity in wild-type and transgenic lines. J6: The 6th line of the 5th generation of transformants; J61: 61th line of the 5th generation of transformants; WT: wild-type. Error bars are means ± SD gotten from (n = 5) five biological replicates. Asterisk indicates the significant difference at *P* values <0.05 between the transgenic lines and the control.

Effect of transgenes on APX, DHAR and GR activities

APX and GR, as well as glutathione, are critical components of the ascorbate-glutathione cycle responsible for the removal of H_2O_2 (Jimenez et al., 1997). Among zthe enzymes of this pathway studied, there are significant differences ($P < 0.05$) between transgenic and WT lines at 0 and 2 days after treatment, however by the end of the experiment (4 days), APX activity of J61 was increased significantly, reaching 4.18 μmol ascorbic acid

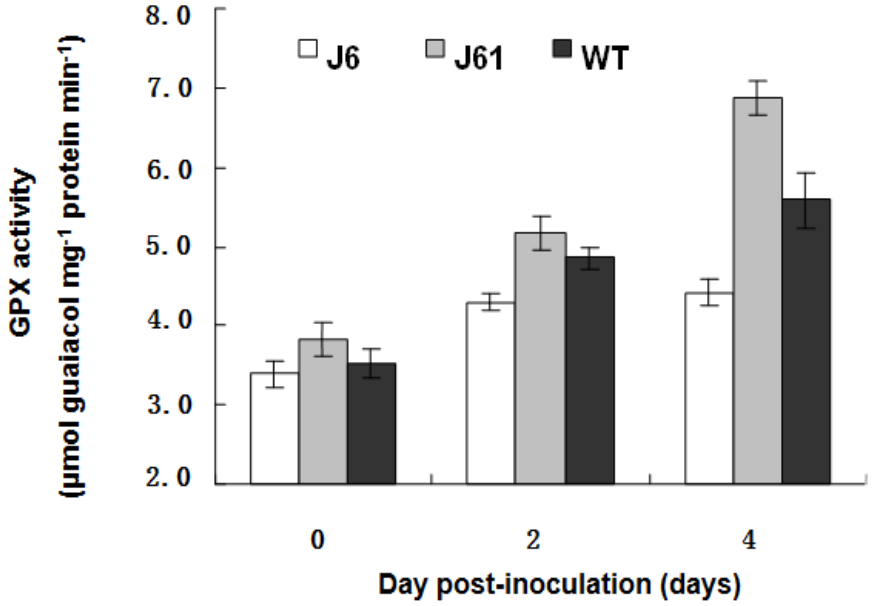

Figure 4. Time-course analysis of GPX activity in wild-type and transgenic lines. J6: The 6th line of the 5th generation of transformants; J61: 61th line of the 5th generation of transformants; WT: wild-type. Error bars are means ± SD gotten from (n = 5) five biological replicates. Asterisk indicates the significant difference at P values <0.05 between the transgenic lines and the control.

(AsA) mg^{-1} protein min^{-1} (Figure 5A). Similarly, the DHAR activity was dramatically increased after invasion (Figure 5B). During time-course, GR activity changed little, except that J6 had a transient dramatic increase at 2 days and thereafter declined sharply to the control level (Figure 5C).

DISCUSSION

Upon pathogen invasion, hosts trigger a defence mechanisms resulting in the generation of reactive oxygen species (ROS), superoxide anion (O_2^-) radicals, hydroxyl ($OH^•$) radicals and hydrogen peroxide (H_2O_2), (Lamb and Dixon, 1997; Azevedo et al., 2008). Increasing evidence reveals that accumulation of ROS severely affects bio-molecules such as proteins, lipids and nucleic acids, leading to oxidative damage at the cellular level (Bertin and Averbeck, 2006; Trachootham et al., 2008). Lipid peroxidation is an indicator of oxidative stress and is estimated as MDA, the principal product of poly-unsaturated fatty acid peroxidation. MDA is mutagenic and carcinogenic, it reacts with DNA and protein to create inter-strand cross-links in DNA forming adducts to deoxyguanosine, deoxyadenosine and deoxycytidine that positively relates to injury of biological membranes (Weber et al., 2004).

MDA levels were shown to increase in *Botrytis cinerea*-elicited maritime pine suspensions (Azevedo et al., 2008), whereas several studies have documented that lipid peroxide levels were unaffected by *B. cinerea* in *Capsicum annuum* fruits, and even decreased in elicited *Arabidopsis* plants (Deighton et al., 1999; Muckenschnabel et al., 2001, 2002). Our present results show that the MDA content increase gradually upon *R. solani* invasion, suggesting that lipid peroxidation might have been induced due to the production of $OH^•$ generated in response to pathogen infection. However, the MDA level was lower in transgenic in comparison with WT plants during infection. Under normal conditions, a low level of ROS is maintained within the organelle. Upon pathogen challenge, ROS producing enzymes (such as NADPH oxidase, cell wall-bound peroxidases, oxalate oxidase, etc) are activated. Plants have developed an intricate and delicate antioxidants defence system to efficiently counteract the toxic effects of ROS, by reducing ROS-induced injuries, aiding in resistance towards stresses.

Intriguingly, our previous studies have revealed that the expression levels of two transgenes varied among the transformants, and transgenic plants possess resistance to pathogen in a dose-dependent manner (data not shown). Our preliminary data where transgenic lines exhibited lower MDA than WT suggests that these responses might be attributed to the expressions of the RCH10 and AGLU1 genes in transgenic plant. One possibility is that RCH10 and AGLU1 function by modulating the expression of other PR-related genes,

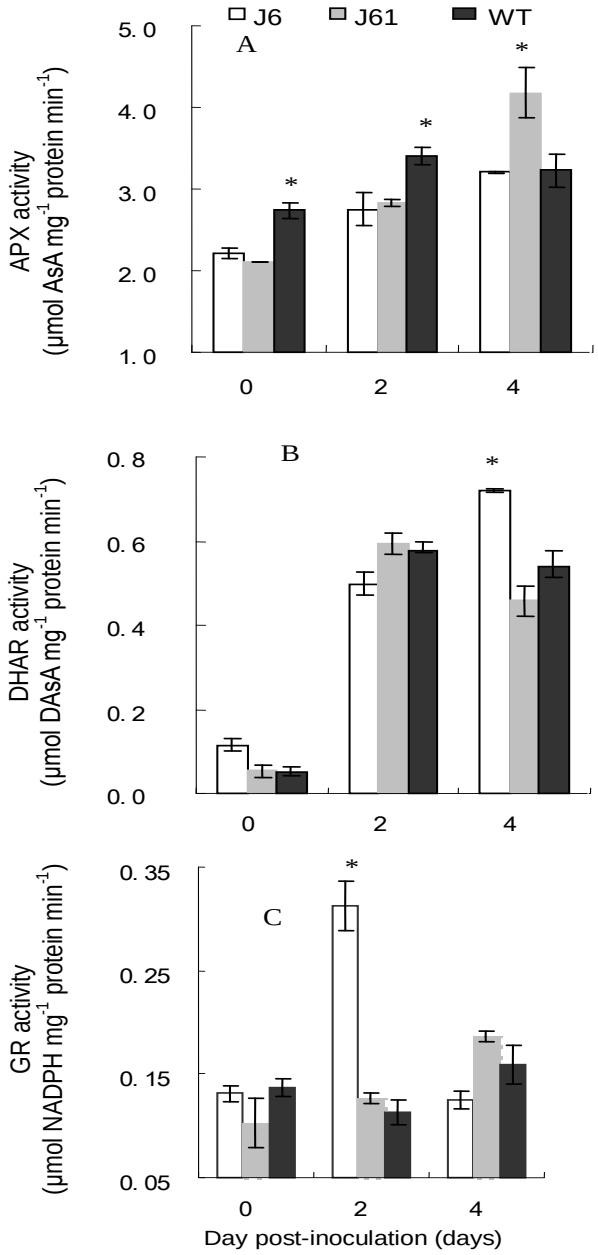

Figure 5. Time-course analysis of APX, DHAR and GR activity in wild-type and transgenic lines. J6: The 6th line of the 5th generation of transformants; J61: 61th line of the 5th generation of transformants; WT: wild-type. Error bars are means ± SD gotten from (n = 5) five biological replicates. Asterisk indicates the significant difference at P values <0.05 between the transgenic lines and the control.

which together, confer synergistic enhanced resistance to oxidative damage via decreasing lipid peroxidation. In other words, RCH10 and AGLU1 assist in ROS scavenging via other pathways apart from lipid oxidation. SOD plays a critical role in catalyzing $O_2^{\cdot-}$ conversion to molecular oxygen and H_2O_2, and is considered as the primary measure to scavenge ROS. Our results demonstrated that upon infecting rice with *R. solani*, mycelium SOD activities were induced and peaked at 2 days after infection after which a decline was observed in WT as compared to transgenic lines (Figure 2). The data presented here on the activity of SOD production in *R. solani*-infected rice are in accordance with *Magnaporthe grisea*-infected rice leaves, where the SOD activity of

incompatibility was higher than that of compatibility within 24 h after treatment. Similar results were obtained from rice exposed to *R. solani* and maritime pine suspension cells exposed to *B. cinerea* spore elicitation (Azevedo et al., 2008). On the contrary, CAT, GPX, APX, DHAR and GR showed no correlation between activity changes and transgenes. Therefore, these results imply that transgenes enhanced resistance to *R. solani* by specifically increasing SOD activity.

Factors that are responsible for the inducible mechanism to resist pathogens invasion in plant are PR, where most of them are regulated by signaling pathways involving one or more of the three regulators jasmonate (JA), ethylene and salicylic acid (SA) (Dong , 1998). However, when expressed in transgenic plants, they reduce only a limited number of disease, depending on the nature of the protein, plant species and pathogens. In summary, our results suggested that the ectopic expression of *RCH10* and *AGLU1* could trigger induction of native *PR* genes expression, which together enhanced resistance to pathogens. This supposition can be tested in future by checking the status of other PR genes in the transgenic plants, and whatever up-regulation of genes is indeed the trigger between RCH10, AGLU1 and pathogens. It is interesting to note that in this case, ROS homeostasis is achieved via specific up-regulation of SOD production, where the rest of the oxidative-related enzymes like APX, DHAR and GR remain unchanged. This suggests that each enzyme is under tight genetic regulation, in this instance, SOD by RCH10 and AGLU1. Therefore, a coordinated gene network is responsible for the production of specific enzyme(s) upon pathogen infestation. Nevertheless, transgene-mediated resistance is a complex process and the underlying mechanisms await more detailed investigation. As such, future experiments should be designed to follow the cascades of changes at the molecular level, revealing particular genes that are regulated to bring out such a significant effect.

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